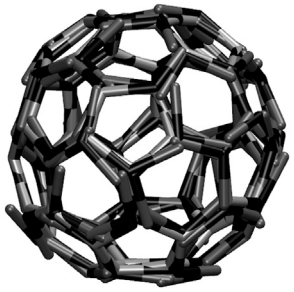
pore, the fraction of the time for which the pore connects the vesicle and SBL membranes (~7-fold increase compared to ~10% for cholesterol free bilayers). Cholesterol also strongly accelerated fusion pore initiation following vesicle docking. With increasing cholesterol concentration, SNAREs open the fusion pore a greater fraction of the time than when SNAREs are absent (~2-3-fold greater with SNAREs than without). Other lipid components tested (PC, PS, PE) had a minimal effect on pore openness. Thus, we find that cholesterol promotes fusion pore opening in SNARE-mediated fusion.

171-Plat
Protein Mobility in Secretory Granules and Fusion Pore Expansion: Factors Affecting Protein Secretion
Annita Ngatchou-Weiss[1], Mary A. Bittner[1], Arun Anantharam[2], Daniel Axelrod[3], Ronald W. Holz[1].
[1]Pharmacology, University of Michigan, Ann Arbor, MI, USA, [2]Biological Sciences, Wayne State University, Detroit, MI, USA, [3]Physics and LSA Biophysics, University of Michigan, Ann Arbor, MI, USA.
We evaluated the importance on post-fusion discharge rates of the mobility of specific lumenal proteins within chromaffin granules in living cells. Two fluorescent proteins were investigated: tissue plasminogen activator (tPA-cerulean), which is discharged over many seconds after fusion, and NPY-cerulean, which is discharged within 200 ms. We developed a method not limited by optical resolution to measure the mobility of fluorescent proteins within individual secretory granules in living chromaffin cells. A bright flash of strongly decaying evanescent field (64 nm exponential decay constant) produced by TIRFM selectively bleached fluorophore that was proximal to the glass coverslip in individual granules (300 nm diameter). Fluorescence recovery occurred as unbleached fluorophore from distal regions of a granule diffused into the proximal regions. This experimental approach is accompanied by a new theoretical, quantitative analysis of recovery that takes into account the evanescent field depth, bleach efficiency, the limited number of total fluorophore molecules in a granule, granule diameter, and duration of bleach. The method and analysis permitted measurement of tPA mobility within chromaffin granules and a comparison of the mobilities of tPA-cerulean and NPY-cerulean. The diffusion coefficient of lumenal tPA-cerulean was 2 x 10^{-10} cm^2/s, ~1/3000 of the expected mobility in aqueous solution. The diffusion of NPY-cerulean was too fast to be resolved. A second goal bearing upon discharge rate was the measurement of fusion pore expansion using polarized TIRFM (Anantharam et al., J.Cell.Biol.2010,188:415-28.). We found that the membrane curvature change due to the fusion pore was much longer lived upon fusion of granules containing tPA-cerulean rather than NPY-cerulean. We quantitatively evaluated the relative roles of low protein mobility within a granule and a restricted fusion pore in determining the release kinetics from individual fused granules. *ANW support NIH fellowship (T32-HL-007853)*

172-Plat
Nanostructure-Induced Membrane Curvature Recruits Endocytosis Machinary in Living Cells
Wenting Zhao[1], Lindsey Hanson[2], Ziliang Lin[3], Yi Cui[1,4], Bianxiao Cui[2].
[1]Materials Science and Engineering, Stanford University, Stanford, CA, USA, [2]Chemistry, Stanford University, Stanford, CA, USA, [3]Applied Physics, Stanford University, Stanford, CA, USA, [4]Stanford Institute for Materials and Energy Sciences, SLAC National Accelerator Laboratory, Menlo Park, CA, USA.
Nanotechnology innovations have advanced biological science by providing new tools for probing cellular process. Whether or how cellular processes are altered upon interacting with such small scale devices are, however, not well understood. One crucial yet overlooked phenomenon is that nanostructures can induce local curvatures on plasma membrane. Modulation of local membrane curvature is known to be important in creating micro-domains for endocytosis initiation.
In the present work, we used electron-beam lithography to make patterned nanopillars arrays with controllable diameters from 60nm to 2000 nm. From both SEM and TEM studies, nanopillars were found to sufficiently induce membrane curvature in living cells. By culturing mammalian cell lines with fluorescent protein labeled clathrin and dynamin on nanopillar substrates, recruitment of these two key proteins in endocytosis machinery were found to preferentially happen on nanopillars in comparison to flat surfaces. Similar phenomenon was also found in adaptor protein AP2 and BAR domain proteins. More interestingly, when changing the nanopillar to other geometries, e.g. nanobar and nanoCUI, such recruitment was found to more correlated with positive and large curvatures. We further studied the dynamics of clathrin and dynamin on nanostructures with gradient geometry, and differential effects were observed. This work provides new insights on the curvature dependent recruitment of endocytosis machinery proteins in living cells, and demonstrates the possibility of using nanofabricated structures as a new platform for membrane curvature manipulation.

173-Plat
A Dynamin Mutant Defines a Super-Constricted Pre-Fission Step
Anna Sunborger[1], Jurgen A. Heyman[1], Shunming Fang[1], Joshua S. Chappie[2], Jenny E. Hinshaw[1].
[1]NIDDK, NIH, Bethesda, MD, USA, [2]Cornell University, Ithaca, NY, USA.
Dynamin is a 100 kDa GTPase that assembles around the necks of invaginated clathrin-coated pits to catalyze membrane fission during the final stages of clathrin-mediated endocytosis. Purified dynamin assembles into helical arrays on lipid templates that resemble the dense collars observed at the necks of clathrin-coated pits in vivo. Recent evidence suggests that the GTP transition state of dynamin serves as a key determinant of productive fission. The transition state stabilizes G domain dimerization, which optimally positions the catalytic machinery and thereby enhancing its intrinsic GTP hydrolysis rate. In the helical array, G domain dimerization between dynamin molecules only occurs between neighboring rungs of the helix. Thus, the architecture of the dynamin polymer ensures that assembly and stimulated turnover are tightly coupled. Here we present a three dimensional structure of a transition-state-defective mutant in the penultimate fission status at 13.5 Å resolution. This structure is tightly constricted with an inner luminal diameter of 4 nm, reaching the theoretical limit required for spontaneous fission. Computational docking of dynamin crystal structures into the 3D reconstruction suggests that a GTP ground state, and not stimulated GTP hydrolysis, drives the dynamin polymer into the super-constricted pre-fission state. Computational docking also positions the proline-rich domain (PRD) close to the G domain, which supports the notion that the PRD can modify the GTPase cycle. The surface accessibility of the PRD allows dynamin partners to bind dynamin throughout its GTPase cycle, and regulate assemble, fission and disassembly.

174-Plat
Clathrin Aggregation by Rotational Brownian Dynamics
Ioana M. Ilie, Wouter K. den Otter, Wim J. Briels.
Twente University, Enschede, Netherlands.
Endo- and exocytosis are processes associated with the transport of nutrients and proteins in to and out of living cells. Upon entering a cell these molecules are collected and encapsulated in vesicles for further transport within the cell. The central protein in the formation process of these vesicles is clathrin. Clathrins consist of three long legs that enable them self-assemble into vesicles and transport cargos within the cell.
We investigate the formation and structure of clathrin cages by means of computer simulations. To achieve this, we developed a highly coarse-grained patchy particle model by representing a clathrin protein as a rigid triskelion with interaction sites on the legs. To simulate their dynamics, we have developed a novel Brownian Dynamics algorithm[1] to describe the realistic motion of the protein. Our algorithm overcomes complications traditionally associated with rotational dynamics of anisotropic particles.



We will show results of the self-assembly of clathrin[1] into cages on a time-scale that is comparable to experimental data. In addition, the simulated cages are structurally similar to those observed by in vitro experiments and the simulations predict the clathrin interaction strength[2].
[1] I.M. Ilie, W.K. denOtter and W.J. Briels, in preparation(2013).
[2] W.K. denOtter and W.J. Briels, Traffic, 12(2011)

175-Plat
Quantifying the Dynamic Interactions between a Clathrin-Coated Pit and Cargo Molecules
Aubrey V. Weigel[1], Michael M. Tamkun[2], **Diego Krapf**[1,3].
[1]School of Biomedical Engineering, Colorado State University, Fort Collins, CO, USA, [2]Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA, [3]Electrical and Computer Engineering, Colorado State University, Fort Collins, CO, USA.
Clathrin-mediated endocytosis is a major pathway of internalization of cargo molecules in eukaryotic cells. This process involves the recruitment of cargo molecules into a growing clathrin-coated pit (CCP). However, cargo-CCP interactions are difficult to study because CCPs display a large degree of lifetime heterogeneity and the interactions with cargo molecules evolve over time. We use single-molecule total internal reflection fluorescence (TIRF) microscopy, in combination with automatic detection and tracking algorithms, to directly visualize the recruitment of individual voltage-gated potassium channels into forming CCPs in living cells. Contrary to widespread ideas, cargo often escapes

from a pit before abortive CCP termination or endocytic vesicle production. Surprisingly, the binding times of cargo molecules associating to CCPs are much shorter than the overall endocytic process. By measuring tens of thousands of capturing events, we build the distribution of capture times and the times that cargo remains confined to a CCP. An analytical stochastic model is developed and compared to the measured distributions. Due to the dynamic nature of the pit, the model is non-Markovian and it displays long-tail power law statistics. Our findings identify one source of the large heterogeneities observed in CCP maturation and provide a mechanism for the anomalous diffusion of proteins in the plasma membrane.

Platform: Cardiac Muscle I

176-Plat

A Revised Atomistic Model of the Cardiac Thin Filament and Application to a Specific Disease Causing Mutation

Michael R. Williams[1], Jil Tardiff[2], Steven Schwartz[1].
[1]Chemistry and Biochemistry, The University of Arizona, Tucson, AZ, USA, [2]Cellular and Molecular Medicine, The University of Arizona, Tucson, AZ, USA.

We have previously proposed an atomistic model of the thin filament which includes the troponin compex (cTn) and tropomyosin (Tm). We here discuss a newly revised model which includes twenty-nine actin subunits, four Tm chains, and the three cTn subunits. In addition, this model includes a corrected region of the Tm overlap based on more recent information. We develop this model to study genetic mutations in the proteins of the cardiac thin filament which can lead to familial hypertrophic cardiomyopathies. When calcium binds to the cardiac troponin C subunit (cTnC), subtle conformational changes propagate through the cTnC to the inhibitor subunit (cTnI), which detaches from actin. The detachment along with conformational propagation through to the cTnT subunit, moves Tm into the open position on actin. When Tm is in the open position, myosin binds to the seven open binding sites on actin, which upon hydrolysis of ATP eventually leads to muscle contraction. Molecular dynamics simulations of the full atomistic model reveal the conformational changes upon calcium activation of the cTnC. Comparison of the wildtype and the Tm D230N mutation sheds light the mutational effects in the Tm overlap region. It is critical to include actin in the model when studying this region. Simulations show mutation results in increased splaying of the Tm C-terminus end in the overlap region, in accord with recent experimental results from one of our labs.

177-Plat

Molecular Mechanism for the Regulation of Cardiac Muscle Contraction by Troponin

Ivanka Sevrieva, Andrea Knowles, **Yin-Biao Sun**.
Randall Division of Cell & Molecular Biophysics, King's College London, London, United Kingdom.

Contraction of cardiac muscle is regulated by Ca^{2+} ions binding to troponin (Tn) in the actin-containing thin filament, leading to a movement of tropomyosin around the filament that allows myosin heads to bind to actin and generate force. However the molecular structural basis of this Tn-mediated signalling pathway has remained obscure. We investigated the conformation of the cardiac Tn on the thin filament and its response to binding of Ca^{2+} to elucidate the molecular mechanism of the regulation of contraction in cardiac muscle. Polarized fluorescence from bifunctional rhodamine was used to determine the orientation of the major component of Tn core domain on the thin filaments of cardiac muscle. We showed that the C-terminus of TnC (CTnC), together with the coiled coil formed by the TnI and TnT chains, did not move during activation and acted as a scaffold that holds N-terminus of TnC (NTnC) and the actin binding regions of troponin I. The NTnC, on the other hand, exhibited multiple orientations during both diastole and systole. By combining the *in situ* orientation data with published *in vitro* measurements of intermolecular distances, we constructed an atomic model for the *in situ* structure of the thin filament that suggests a plausible molecular mechanism for the regulation of heart muscle.

178-Plat

Calcium-Sensitive Dynamic Effects of Troponin's TnI Inhibitory Region

Julie Mouannes Kozaili, Devanand Kowlessur, **Larry S. Tobacman**.
Medicine, University of Illinois at Chicago, Chicago, IL, USA.

Contraction of cardiac and skeletal muscle is tightly controlled by the thin filament protein troponin, which acts as an off switch when the Ca^{2+} concentration is low. We previously studied, by mutational replacements, the function of an 11-residue segment in the TnI subunit that is called the inhibitory peptide (Ip) because of its potency when studied as an isolated oligomer. Ip replacement with gly-ala linkers selectively altered specific functional properties of the thin filament. The present work further investigates the role of Ip and Ip replacement, by quantitative mapping of troponin dynamics using hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS). Effects of Ip replacement were assessed in the presence and absence of TnC regulatory site II Ca^{2+}. In the absence of Ca^{2+}, the Ip-mutation was widely destabilizing, selectively affecting many regions both near and far from Ip. Ca^{2+} broadly but incompletely reversed this effect. After Ip replacement, Ca^{2+} stabilized the TnT-TnI coiled coil, parts of TnI helix 1, and the N-lobe of TnC, but not the switch helix of TnI. The biggest effects were observed in the TnT-TnI coiled coil region, helix 1 and helix 4 of TnI and parts of TnC's N and C lobes. In presence of Ca^{2+}, the Ip-mutation caused more mixed effects, with helix 4 of TnI destabilized, a mixed effect on the TnI regions contained in the coiled coil and helix 1 as well as the N and C lobes of TnC. In both cases (with and without Ca^{2+}), an unexpected stabilization toward the C-term of TnI was observed. The results emphasize the importance of the Ip region both in the "on" and "off" states of troponin. Unexpected effects of Ip replacement with a flexible linker propagate across the Tn molecule.

179-Plat

Dynamic Effects of Tropomyosin D230N Mutation and Fetal Troponin T on the Tropomyosin Overlap Region

Mark T. McConnell[1], Jayant J. Jayasundar[2], Lauren Grinspan[3], Ofer Z. Fass[2], Benjamin Schwartz[2], Jil C. Tardiff[2].
[1]Biomedical Engineering, University of Arizona, Tucson, AZ, USA, [2]Cellular and Molecular Medicine, University of Arizona, Tucson, AZ, USA, [3]Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY, USA.

Introduction: Mutations in the cardiac thin filament cause changes in protein structure and dynamics. These alterations result in the complex tissue remodeling seen in patients with hypertrophic and dilated cardiomyopathies. Our group is investigating the effects of these mutations on the tropomyosin (Tm) head-to-tail overlap domain. In particular, we are investigating the alpha-Tm D230N mutation that causes a unique bimodal distribution of remodeling. We hypothesize that this is caused by the modulatory effects of the fetal form of cTnT (cTnT1) on overlap structure and function.
Methods: Tm was modified to have one cysteine at residue 271 for both wildtype and D230N mutations, and was labeled with the FRET acceptor DDPM. cTnT1 or adult cardiac troponin T (cTnT3) were modified to have one cysteine at residue 100 and labeled with the FRET donor AEDANS. Labeled cTnT1 or cTnT3 were reconstituted into the troponin complex and combined with labeled Tm of either wildtype or D230N. Steady-state and lifetime data was collected.
Results: The distance between the labeled sites in the wildtype complex increased with calcium activation. In contrast, the calcium activated state of the D230N complex resulted in a shorter measured distance. cTnT1 also resulted in a closer distance. cTnT1 in addition to D230N mutation resulted in a closer orientation compared D230N or cTnT1 alone. Differences in measured distances caused by the variants were more pronounced in the calcium activated state than in the low calcium state.
Conclusion: The increased distance measured with wildtype complex supported the increased flexibility of Tm accompanying thin filament activation. The decreased distance measured at the Tm overlap supports the hypothesis that cTnT1 interacts with D230N in a manner that amplifies the effects of the mutation alone at the Tm overlap region.

180-Plat

In Situ Structural Changes in Thick and Thin Filaments of Cardiac Muscle Induced by Fragments of Myosin Binding Protein C (MyBP-C)

Thomas Kampourakis, Yin-Biao Sun, Mathias Gautel, Malcolm Irving.
Randall Division for Cell and Molecular Biophysics, King's College London, London, United Kingdom.

MyBP-C is a component of the thick filaments of striated muscle, and mutations in the cardiac MyBP-C gene are the second most common cause of hypertrophic cardiomyopathy. The C-terminus of MyBP-C is bound to the thick filament, but the N-terminus is believed to interact with both the thin filaments and the S2 domain of myosin in the thick filaments. These interactions are phosphorylation-dependent, and are thought to regulate cardiac contractility. In this study, polarized fluorescence was used to monitor structural changes of the cardiac myosin regulatory light chain (cRLC) in the thick filaments and cardiac troponin C (cTnC) in the thin filaments on activation of skinned cardiac muscle either by calcium or by exogenous N-terminal fragments of cardiac MyBP-C. The structural changes in both proteins produced by activation with 50 μM C1-C2 fragment of cMyBPC at sarcomere length 2.1 μm, 20°C, pCa 9 were similar to those produced by calcium activation (pCa 4.5). However, only the cTnC probes showed significant structural changes when contraction was inhibited by blebbistatin, suggesting that the activating effect of C1-C2 is mediated by binding to the thin filament.
Supported by the British Heart Foundation